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HIV-1 Tat targets Tip60 to impair the apoptotic cell response to genotoxic stresses

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HIV-1 transactivator Tat uses cellular acetylation signalling by targeting several cellular histone acetyltransferases (HAT) to optimize its various functions. Although Tip60 was the first HAT identified to interact with Tat, the biological significance of this interaction has remained obscure. We had previously shown that Tat represses Tip60 HAT activity. Here, a new mechanism of Tip60 neutralization by Tat is described, where Tip60 is identified as a substrate for the newly reported p300/CBP-associated E4-type ubiquitin-ligase activity, and Tat uses this mechanism to induce the polyubiquitination and degradation of Tip60. Tip60 targeting by Tat results in a dramatic impairment of the Tip60-dependent apoptotic cell response to DNA damage. These data reveal yet unknown strategies developed by HIV-1 to increase cell resistance to genotoxic stresses and show a role of Tat as a modulator of cellular protein ubiquitination.

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Introduction

The activity of p53 as a key factor mediating cell response to DNA damage largely relies on its role as a sequence-specific transcription factor, inducing the expression of genes involved in the control of cell cycle, apoptosis and DNA repair. Moreover, this activity of p53 depends on a complex interplay between several signalling pathways, which control its post-translational modifications and consequently modulate its diverse properties, including DNA binding, subcellular localization and stability (Brooks and Gu, 2003; Oren, 2003). One of the modulators of p53 function is the ubiquitin ligase, Mdm2, which not only inactivates p53 but also induces its degradation through the proteasome (Michael and Oren, 2003; Moll and Petrenko, 2003). We have recently found that Mdm2 also interacts with and modulates the stability of another cellular factor, the histone acetyltransferase (HAT) Tip60 (Legube *et al*, 2002). Additionally, these data have shown that, like p53, Tip60 accumulates in cells in response to UV irradiation, raising the possibility that both proteins could act in similar regulatory pathways. This hypothesis has recently received remarkable supporting evidence. Indeed, a large-scale RNAi screen in human cells has shown that Tip60 is a key component in the antiproliferative cell response (Berns *et al*, 2004). Downregulation of Tip60 causes a strong reduction of $p21^{cip1}$ (mRNA and protein) and, conversely, the overexpression of Tip60 enhances p21 expression and stimulates the G_1 cell cycle arrest in response to DNA damage. These data highlight a role for Tip60 in the cell response to DNA damage. Indeed, such a role for Tip60, which had been proposed after early functional analysis of the Tip60 complex (Ikura *et al*, 2000), was recently confirmed in *Drosophila*. Indeed, dTip60 and its associated proteins have been shown to mediate the replacement of *Drosophila* phosphorylated H2AX homolog by a nonmodified protein after DNA damage (Kusch *et al*, 2004).

These functional similarities between Tip60 and p53 could explain why cells use a common regulatory mechanism (involving Mdm2) to control the cellular concentration of both proteins. The need to balance the concentration of these two factors also explains the existence of a sensor mechanism linking the control of the cellular concentration of p53 to that of Tip60 (Legube *et al*, 2004).

Finally, Tip60 also seems to play a role in E2F-dependent transcriptional activation at the S-phase entry, since the activating E2F transcription factors were proposed to recruit Tip60 to the E2F-responsive gene promoters (Taubert *et al*, 2004).

Since p53 is a known target of several viral proteins inducing its degradation, and because of the participation of both p53 and Tip60 in the same cell response pathways, one might expect Tip60 to be also specifically targeted and inactivated by viral proteins. Interestingly, Tip60 was initially discovered as a partner of the HIV-1 transactivator Tat (Kamine *et al*, 1996). Besides Tip60, Tat is also capable of

recruiting at least five other cellular HATs: CBP, p300, P/CAF, GCN5 and $TAF_{II}250$. The consequence of the targeting of most of the cellular HATs by Tat is a considerable gain of function for the viral protein, since Tat is itself acetylated by at least four of them (CBP, p300, P/CAF and GCN5), optimizing its transactivator activity (Nakatani, 2002; Caron *et al*, 2003; Ott *et al*, 2004). In contrast to other HATs, Tat is not a substrate for Tip60 (our unpublished data). Our previous investigations have shown that Tat is able to inhibit the HAT activity of Tip60 (Creaven *et al*, 1999), suggesting that the activity of Tip60 could be undesirable for proper HIV-1 replication and propagation (Caron *et al*, 2003). However, the significance of Tip60 targeting by Tat has remained unclear.

Here, the functional significance of the targeting of Tip60 by Tat is shown for the first time and new aspects of Tip60 neutralization by Tat are unravelled. Our data show that, besides inactivating Tip60 catalytic activity, Tat is able to induce a selective degradation of the protein. Taking advantage of the striking similarities between p53 and Tip60 regulation, we unravel the mechanism of Tat-mediated degradation of Tip60. Indeed, p300 and CBP were recently shown to stimulate p53 polyubiquitination through an associated ubiquitin-ligase E4-type activity (Grossman *et al*, 2003). This mechanism is used by Tat to enhance Tip60 polyubiquitination. Our data therefore confirm the p300/CBP E4-type activity by identifying Tip60 as its second substrate, and show for the first time that Tat uses this activity to induce the degradation of a specific cellular factor. Furthermore, here we show that Tip60 plays a critical role in controlling the cell response to genotoxic treatments, and that its Tat-mediated inactivation and degradation give the cells a remarkable resistance to stress-induced apoptosis. These data point to Tip60 as an important cellular target of HIV-1.

Results

Tat accelerates Tip60 turnover

During our previous investigations, we had noticed that the coexpression of Tat reproducibly caused a decrease in Tip60 accumulation. To test if Tat could modulate the stability of Tip60, the protein was transiently expressed in Cos cells in the presence or absence of Tat, and cells were treated with the widely used inhibitor of translation cycloheximide (CX) for different periods of time. The results showed that Tat expression is associated with a significant acceleration in Tip60 decay compared to Tip60 alone (Figure 1A, Tip60 panel, compare –Tat with +Tat). The half-life of p300, another Tat-interacting HAT, was not affected by Tat coexpression. Since it is known that Tip60 is degraded by the ubiquitin/proteasome system (Legube *et al*, 2002), we wondered whether Tat could stimulate its polyubiquitination. Ha-tagged Tip60 and six histidine-tagged ubiquitin (His-Ub) were expressed in the absence or presence of Tat, and Tip60 ubiquitination was estimated by capturing His-Ub from cell extracts with nickel beads under denaturing conditions, and

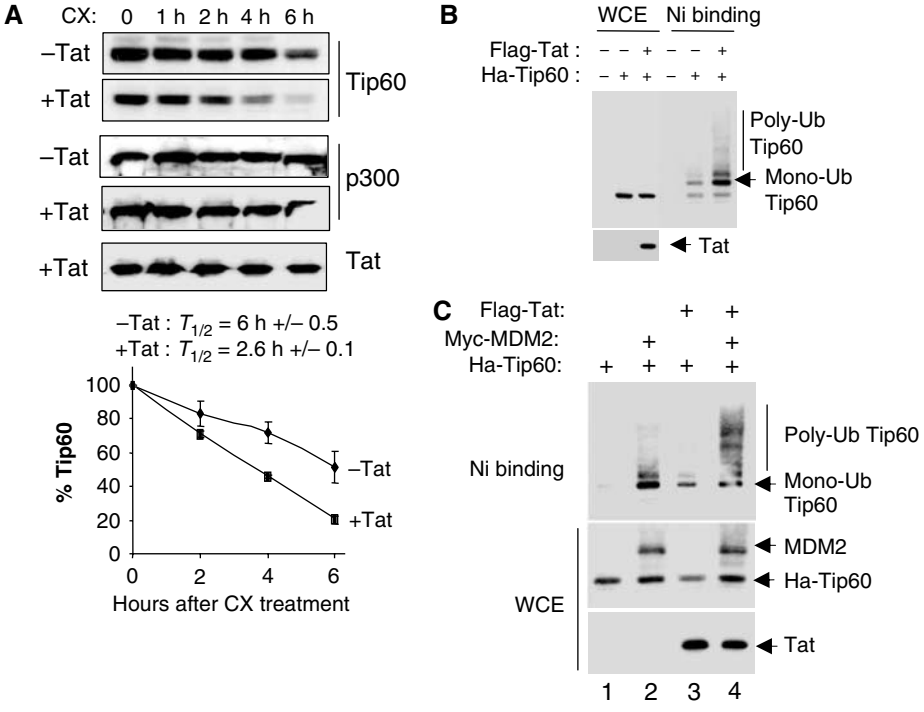

Figure 1 Tat induces the polyubiquitination and degradation of Tip60. (**A**) Cos cells were transfected with Ha-tagged Tip60 or Myc-tagged p300 expression vectors (Tip60 and p300 panels, respectively), in the presence (+Tat) or absence (−Tat) of Flag-Tat expression vectors, and treated with CX for the indicated times. Tip60 half-life was measured and averaged from three different assays using the densitometric signals from the Western blots. Tat and p300 panels show the stability of both proteins during CX treatment. Tip60, p300 and Tat were detected on Western blots with anti-Ha, anti-Myc and anti-Flag antibodies, respectively. (**B**) Cos cells were cotransfected with expression vectors for Ha-Tip60 and His-tagged ubiquitin in the presence or absence of Flag-Tat expression and the ubiquitinated Tip60 was retained on nickel beads and analysed by an anti-Ha Western blot. (**C**) Cos cells were cotransfected with expression vectors for His-Ub and Ha-Tip60 in the presence of Flag-Tat or Mdm2 or both, the level of Tip60 ubiquitination was monitored as in panel B (upper panel). (B, C) WCE panels show the expression of ectopically expressed proteins in whole-cell extracts, detected by Western blot using anti-Flag (Tat), anti-Mdm2 or anti-Ha (Tip60) antibodies.

detecting ubiquitinated Tip60 by an anti-HA antibody. This assay revealed a stronger ubiquitination of Tip60 in the presence of Tat (Figure 1B).

We had previously established that Tip60 is ubiquitinated by the E3 ubiquitin-ligase Mdm2 (Legube *et al*, 2002). The nickel capture assay was used to evaluate the effect of Tat on Mdm2-dependent Tip60 ubiquitination. Figure 1C shows that the expression of Mdm2 stimulated the accumulation of the ubiquitinated forms of Tip60 (lane 2). Interestingly, the additional expression of Tat strongly enhanced the accumulation of the polyubiquitinated forms of Tip60 (compare lane 4 to 2).

Tat uses p300/CBP to stimulate Tip60 polyubiquitination

The experiments described above confirm that Mdm2 mediates the ubiquitination of Tip60 and show that Tat stimulates the polyubiquitin chains formation. This suggested that Tat could use a cellular E4-type activity to mediate Tip60 polyubiquitination. A recent work has reported that CBP and p300 have an E4 activity and act in concert with Mdm2 to polyubiquitinate p53 (Grossman *et al*, 2003). Since CBP and p300 are Tat-interacting proteins, we then hypothesized that Tat may use these proteins to polyubiquitinate Tip60. To show the involvement of p300/CBP in the Tip60 polyubiquitination, either p300 or CBP was expressed in the absence or presence of Tat, and a nickel-binding assay was performed. Figure 2A shows that both p300 (left panel) and CBP (right panel) strongly stimulate the effect of Tat on Tip60 polyubiquitination (Figure 2A, compare lane 4 with 3 for p300, and lane 8 with 6 for CBP). The involvement of p300 in the Tat-mediated stimulation of Tip60 ubiquitination was further confirmed by monitoring the Tip60 ubiquitination in the presence of Tat after the downregulation of the endogenous p300 by siRNA (Figure 2B). Mdm2 and p300 could therefore cooperate to polyubiquitinate Tip60. In order to confirm this hypothesis, Mdm2 and p300 were coexpressed and Tip60 ubiquitination was monitored. Figure 2C shows that the coexpression of p300 and Mdm2 strongly stimulates Tip60 polyubiquitination compared to either p300 or Mdm2 alone.

It appeared important to check whether Tat was capable of stimulating the cooperative action of p300 and Mdm2 in Tip60 ubiquitination. In order to visualize the stimulatory effects of Tat, suboptimal amounts of Mdm2 and p300 were expressed in cells in the absence or presence of Tat (Figure 2C, compare lanes 5 and 6). This experiment clearly showed that Tat could enhance the activity of p300–Mdm2

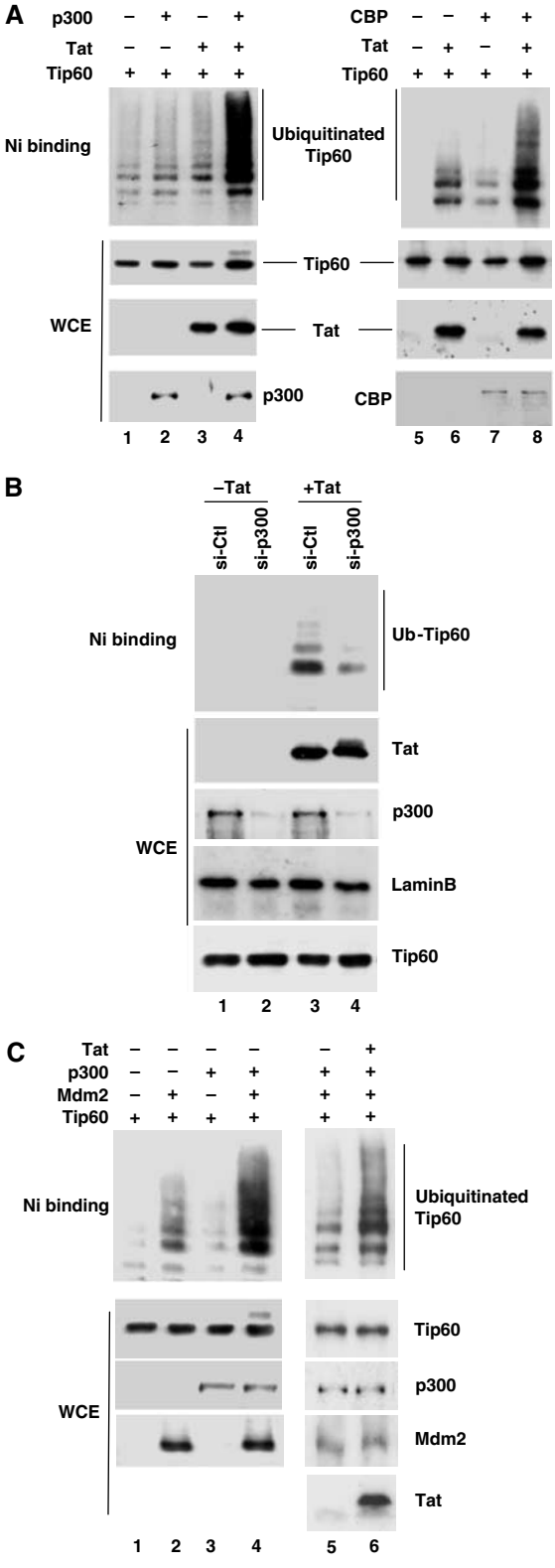

Figure 2 Tat uses p300/CBP to induce the polyubiquitination of Tip60. (**A**) Tip60 ubiquitination was monitored as in Figure 1B in the presence of Myc-p300, Flag-Tat or both (left panel), or Ha-CBP, Flag-Tat or both (right panel). WCE panels show the expression, in whole-cell extracts, of ectopically expressed proteins, detected using anti-Flag (Tat), anti Myc (p300) or anti-Ha (Tip60, CBP) antibodies, respectively. (**B**) Human H1299 cells were treated with siRNA targeting p300 (si-p300) or a control (si-Ctl) siRNA, transfected with Ha-Tip60 and His-ubiquitin, in the presence (+Tat) or absence (−Tat) of Flag-Tat. Ubiquitinated Ha-Tip60 was analysed by the nickel pull-down assay, and Ha-Tip60, Flag-Tat, endogenous p300 and lamin B (the latter to control that equivalent amounts of total extracts were loaded) were analysed by Western blots. (**C**) Tip60 ubiquitination was monitored as above in the presence of Myc-p300, Mdm2 or both (lanes 1–4). The stimulatory effect of Tat on the cooperative action of p300-Mdm2 on Tip60 ubiquitination was monitored by expressing the two proteins in the absence or presence of Tat (lanes 5 and 6). In this experiment, in order to visualize the effect of Tat, the cooperative action of p300-Mdm2 had to be reduced by expressing less Mdm2 and p300 (cells were transfected by a third of the amount of Mdm2 and a half the amount of p300 expression vectors used in experiments shown in lanes 2 and 4).

in Tip60 ubiquitination. It therefore confirmed our original hypothesis on the ability of Tat to use the p300–Mdm2 machinery to stimulate Tip60 degradation. It is noteworthy that, in all experiments involving His-Ub overexpression, we noticed an attenuation of Tip60 downregulation by Tat or other factors such as Mdm2. One explanation could be an increased competition between other ubiquitinated cellular proteins and Tip60 for degradation by the proteasome.

Tip60 interacts with and is acetylated by p300/CBP

Because of several apparent functional (Berns *et al*, 2004) and regulatory (Legube *et al*, 2004) similarities between p53 and Tip60, we wondered if Tip60 could, like p53, interact with or be acetylated by p300/CBP. Indeed, p53 is known to be a partner of p300 and CBP as well as a substrate of their HAT activity (Grossman, 2001). Moreover, it was shown that p53 acetylation controls its stability (Ito *et al*, 2002).

The interaction of Tip60 with CBP was tested by co-immunoprecipitation from cell extracts expressing Ha-Tip60 (full length or deletion mutants; Figure 3A) and a Gal4-fused CBP fragment (amino acids 1099–1758, encompassing its bromo- and HAT domains). Figure 3B shows that wild-type (wt) Tip60 interacts with CBP in cells, while neither the 211 N-terminal amino acids of Tip60 encompassing its chromo-domain, nor its C-terminal domain (amino acids 365–511) could bind the CBP fragment used in this experiment (Figure 3B, panels 2 and 5). We concluded from these data that a region corresponding to amino acids 212–364, and containing the zinc-finger of Tip60, is involved in the interaction. The interaction between Tip60 and p300 was also observed between the full-length p300 and full-length Tip60 (Figure 3C, panel 1). Here, we also confirmed that the p300/CBP interaction domain of Tip60 excludes the N-terminal 211 and the C-terminal 147 amino-acid regions of Tip60 (Figure 3C, panels 2 and 3). Finally, after the immunoprecipitation of the

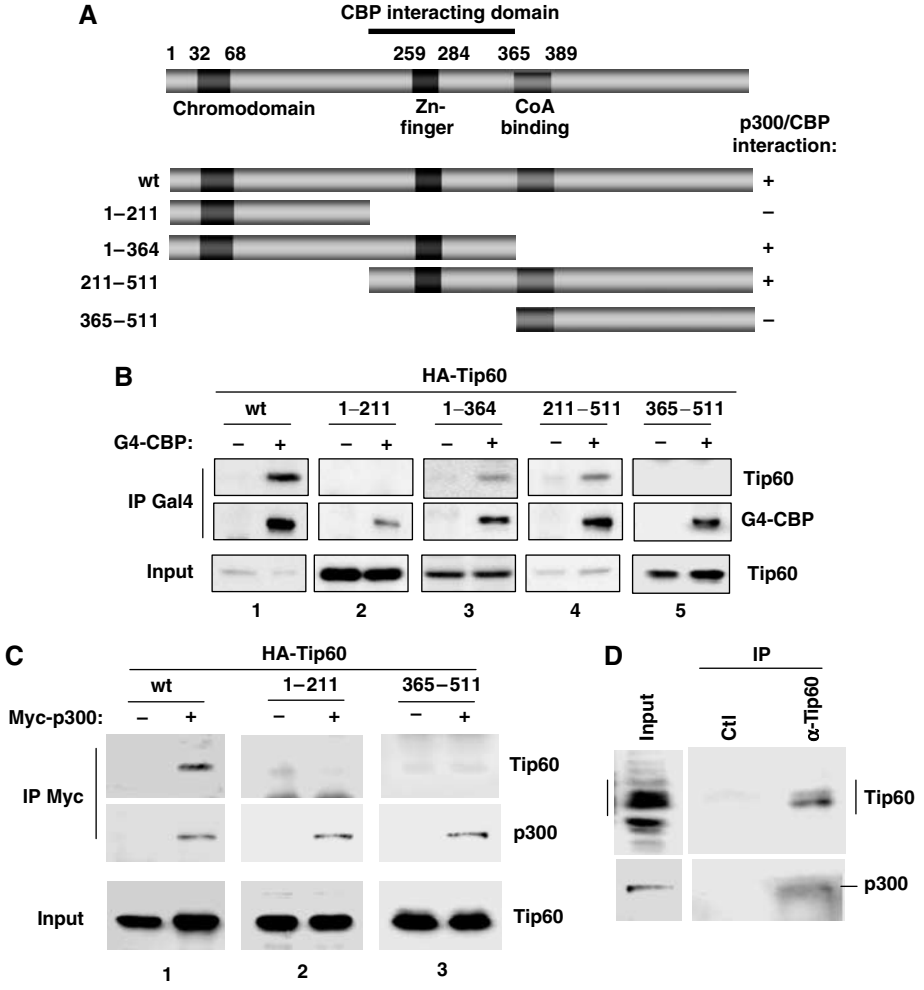

Figure 3 Tip60 interacts with CBP. (**A**) Schematic representation of wild-type (wt) and deletion mutants of Tip60. (**B**) Ha-tagged Tip60 and the indicated mutants of Tip60 were expressed in Cos cells in the absence or presence of Gal4-CBP, encompassing the 1099–1758 amino-acid region of CBP, which contains its HAT domain, fused to the DNA-binding domain of Gal4. After anti-Gal4 immunoprecipitation, Gal4-CBP and associated Tip60 fragments were detected (anti-Gal4 and anti-Ha Western blots, respectively). The input panel shows the amount of Tip60 fragments expressed (anti-Ha Western blot). (**C**) Ha-tagged Tip60 and its indicated mutants were expressed in Cos cells in the absence or presence of Myc-tagged full-length p300 (Myc-p300). After anti-Myc immunoprecipitation, p300 and associated Tip60 fragments were detected (anti-Myc and anti-Ha Western blots, respectively). The input panel shows the amount of Tip60 fragments expressed (anti-Ha Western blot). (**D**) Endogenous Tip60 was immunoprecipitated from nuclear extracts of Jurkat cells, with a goat anti-Tip60 antibody (α-Tip60) or an irrelevant goat serum as negative control (Ctl). Tip60 and p300 were revealed by Western blots using rabbit anti-Tip60 and anti-p300 antibodies, respectively.

endogenous Tip60, we observed the co-immunoprecipitation of a fraction of the endogenous p300 in Jurkat cells (Figure 3D). Note that the anti-Tip60 antibody, while detecting different Tip60-related bands in whole-cell extracts (Frank *et al*, 2003), immunoprecipitates only the long forms of Tip60, suggesting that different Tip60 species are not equally recognized by the antibody in the extracts. These Tip60 isoforms are either Tip60 splice variants (Ran and Pereira-Smith, 2000; Sheridan *et al*, 2001; Legube and Trouche, 2003) or post-translationally modified forms of the protein (Lemercier *et al*, 2003).

The possible acetylation of Tip60 by p300/CBP was then monitored by probing immunoprecipitated Tip60 after its coexpression with either CBP or p300 with an anti-acetylated

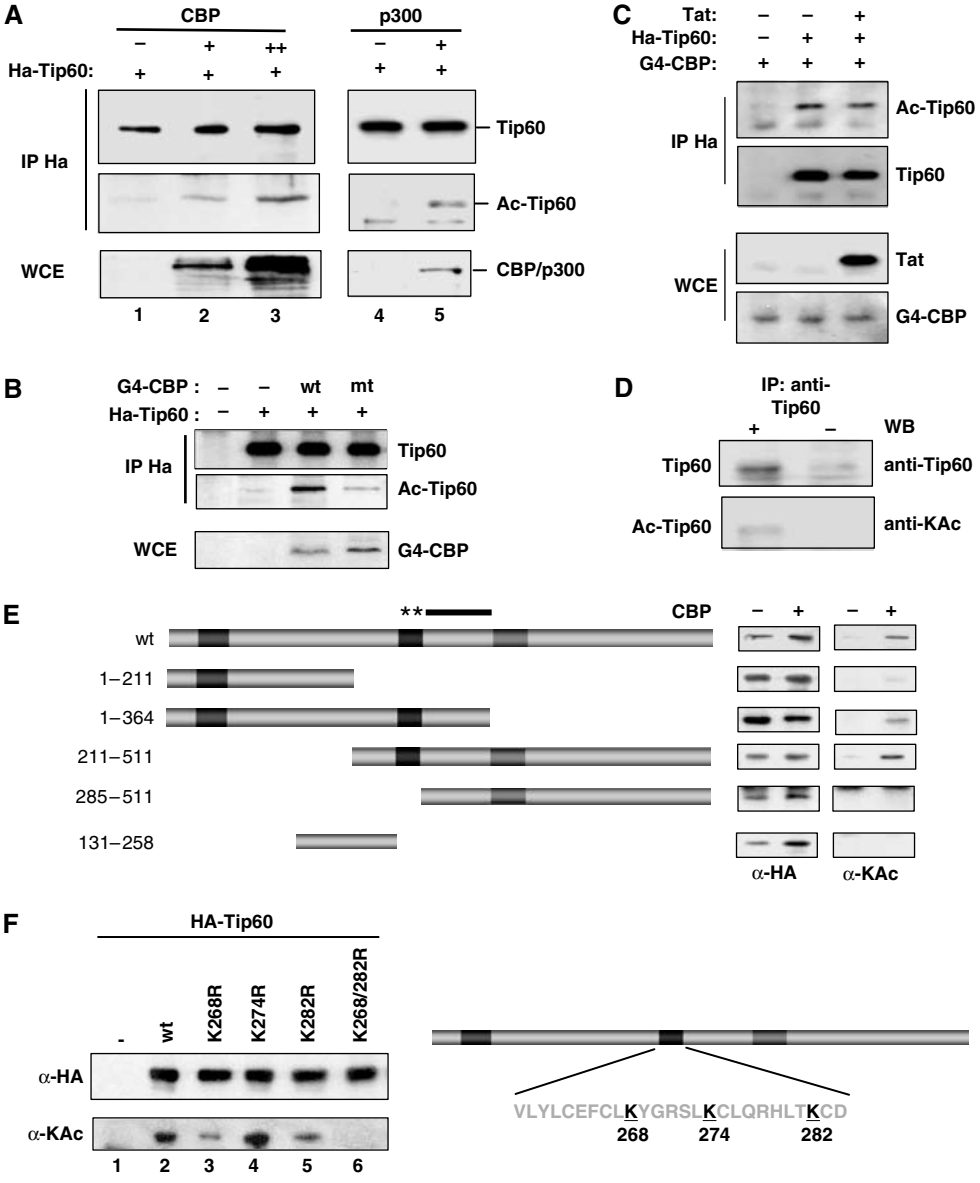

Figure 4 Tip60 is a substrate for the HAT activity of p300/CBP. (**A**) Cos cells were cotransfected with Ha-Tip60 expression vector and increasing amounts of CBP (+, ++) or p300. Tip60 was immunoprecipitated after treating cells with trichostatin A (TSA), and revealed either by an anti-Ha (Tip60) or an anti-acetylated lysine (Ac-Tip60). The amounts of expressed CBP or p300 are shown in the WCE panel. (**B**) The experiment was performed as in panel A, except that Ha-Tip60 was expressed with Gal4-CBP either wild type (wt) or inactivated by a mutation (mt) in its HAT domain. Tip60 was immunoprecipitated as above and its acetylation was examined. The WCE panel shows the expression of Gal4 fusion proteins. (**C**) The experiment was performed as in panel A, except that Ha-Tip60 was expressed in the presence or absence of Flag-tagged Tat, as indicated. The WCE panels show the expression of Gal4-CBP (anti-Gal4 Western blot) and Tat (anti-Flag Western blot). (**D**) Tip60 was immunoprecipitated from Jurkat cell nuclear extracts using an anti-Tip60 serum (+) or a nonimmune serum (−), and the presence of Tip60 was detected first using the anti-acetylated lysine antibody (lower panel) and then an anti-Tip60 antibody (upper panel). (**E**) Wild-type Tip60 and the indicated fragments were expressed in Cos cells in the absence (−) or presence (+) of ectopically expressed CBP, immunoprecipitated and proteins revealed first by an anti-acetylated lysine antibody (α-KAc, right) and then by an anti-Ha (Tip60 fragments, left). (**F**) The three underlined lysines (K) in the 259–284 region of Tip60 were individually replaced by arginines (R) (K268R, K274R and K282R, respectively). In the K268/282R mutant, the two indicated K are replaced by R. Wild-type Tip60 or the indicated mutants were expressed in Cos cells in the presence of CBP and, after anti-Ha immunoprecipitation (controlled in α-Ha panel), their acetylation was monitored as in panel A (α-KAc panel).

lysine antibody. Figure 4A shows that a fraction of the ectopically expressed Tip60 is acetylated and that the coexpression of both full-length CBP and p300 strongly increases the proportion of acetylated Tip60 (Figure 4A, lanes 2, 3 and 5). To ensure that the acetyltransferase activity of CBP is indeed responsible for this modification, Tip60 acetylation was analysed in the presence of a truncated form of CBP (G4-CBP used in Figure 3B) containing its HAT domain, either wild type or inactivated by a punctual mutation (Martinez-Balbas *et al*, 1998). The results showed that Tip60 can be acetylated only by the wild-type but not the inactive form of CBP HAT domain (Figure 4B). Interestingly, the acetylation of Tip60 by CBP was not modified in the presence of Tat (Figure 4C, and not shown). This observation extends our previous findings that Tat had no effect on the p300/CBP-dependent acetylation of p53 or MyoD (Col *et al*, 2002). The use of Jurkat cells allowed us to show that the immunoprecipitated endogenous Tip60 was also recognized by the anti-acetylated lysine antibody, indicating that at least a fraction of the endogenous Tip60 in these cells is acetylated (Figure 4D). Altogether, our results point to Tip60 as a new acetylated cellular factor.

We then mapped the target lysines of Tip60, by testing the ability of several Tip60 deletion mutants to be acetylated in the presence of CBP. Figure 4E shows that the presence of the zinc-finger region (aa 259–284) is required for efficient acetylation of Tip60 by CBP. This region contains three lysines, at positions 268, 274 and 282, which were replaced by an arginine in the full-length Tip60, and the level of acetylation of each of these mutants was analysed. A mutation of K274 did not abolish the acetylation level of Tip60 (Figure 4F, lane 4), whereas a mutation of either K268 or K282 significantly reduced it (Figure 4F, lanes 3 and 5). No acetylation was detectable when K268 and K282 where mutated together (Figure 4F, lane 6), confirming that lysines 268 and 282 of Tip60 are both targeted for acetylation by p300/CBP in the cell.

p300/CBP-dependent stimulation of Tip60 ubiquitination is independent of Tip60 acetylation and p300/CBP HAT activity

Interestingly, evidence is accumulating in the literature, which shows that protein ubiquitination can be modulated by acetylation (Caron *et al*, 2005). In order to evaluate the relationship between Tip60 acetylation and ubiquitination, it appeared important to determine if ubiquitination and acetylation could target the same lysines of Tip60 or not. We therefore mapped the region of the protein that bears the ubiquitinated lysines.

Several deletion mutants of Tip60 were expressed in the presence of His-Ub, with or without p300 and Tat, and their ubiquitination level was monitored as previously described. While Tip60 mutants 1–211 and 365–511, incapable of interacting with p300/CBP, could not be ubiquitinated (Figure 5A, panels 2 and 6), the mutants corresponding to amino acids 1–364, 211–511 and 285–511 of the protein were efficiently ubiquitinated, and the level of ubiquitination was highly stimulated in the presence of p300 and Tat (Figure 5A, panels 3–5). We concluded from this assay that the domain of Tip60 ubiquitination is located between amino acids 285 and 364, beyond the acetylation domain determined above. These results suggest that acetylation and ubiquitination target different but neighbouring domains of Tip60 (indicated in Figure 4E).

To further test if ubiquitination of Tip60 depends on its acetylation state, the ubiquitination assay was performed using the non-acetyl-acceptor Tip60 K268/282R mutant, in the presence or absence of p300 and Tat. This mutant was polyubiquitinated as efficiently as the wild-type protein, indicating that the efficiency of polyubiquitination is independent of Tip60 acetylation (Figure 5B). This conclusion was further supported by the observation that Tat could also use a mutant of p300 containing an inactive HAT domain to stimulate the polyubiquitination of Tip60 (Figure 5C). In contrast, a p300 mutant (p300 ΔN) deleted of its N-terminal 870 amino acids, previously reported for having lost its E4 activity (Grossman *et al*, 2003), was completely unable to induce the polyubiquitination of Tip60, in the presence of Tat (Figure 5D). Interestingly, p300 ΔN interacted as efficiently as the full-length p300 with Tip60 (Figure 5E).

Altogether, these experiments demonstrate that the interaction between Tip60 and p300 is indispensable for the Tat-mediated Tip60 ubiquitination (Figure 5A). They also show that the interaction between the two proteins alone is not sufficient and that the presence of the N-terminal domain of p300 is also very important. We therefore conclude that the polyubiquitination of Tip60 induced by Tat is entirely dependent on the E4, but not the HAT, activity of p300.

Tat-induced neutralization of Tip60 inhibits the apoptotic response of cells to a genotoxic treatment

A series of experiments was planned to investigate the functional significance of the targeting of Tip60 by Tat. Our hypothesis was that the Tat-mediated inactivation of Tip60 could inhibit the apoptotic response of cells to genotoxic treatments, in which Tip60 had been shown to be involved (Ikura *et al*, 2000; Kusch *et al*, 2004).

A cell line stably expressing Tat was established. To avoid a possible functional redundancy between Tip60 and p53, we chose a p53-deficient human cell line, H1299, expressing detectable amounts of Tip60 (Figure 6A, lower panel, lane 1). We controlled for the fact that the cells were capable of undergoing apoptosis in response to the genotoxic reagent actinomycin D (Act D; Figure 6A, and not shown). Clones stably expressing Tat were then tested to monitor the effect of Tat expression on the amount of endogenous Tip60 present in these cells. Figure 6A shows that (1) our clones are constituted of a homogenous population of Tat-expressing cells (one example is shown in the upper panel) and (2) Tat expression leads to a downregulation of endogenous Tip60 (lower panel, two independent Tat-expressing clones are analysed, tracks 2 and 3). One of the Tat-expressing clones was then challenged with different doses of Act D, and the apoptotic cells were visualized by monitoring the appearance of activated caspase 3. Figure 6A shows that the expression of Tat gives cells a remarkable resistance to induced apoptosis compared to the control cell line. Considering the important role of Tip60 in linking DNA damage to an apoptotic response, we hypothesized that the inactivation and downregulation of Tip60 by Tat could participate in the observed resistance of cells to Act D treatment. To prove that Tip60 is indeed necessary for the apoptotic response after Act D treatment, the protein was downregulated using an appro-

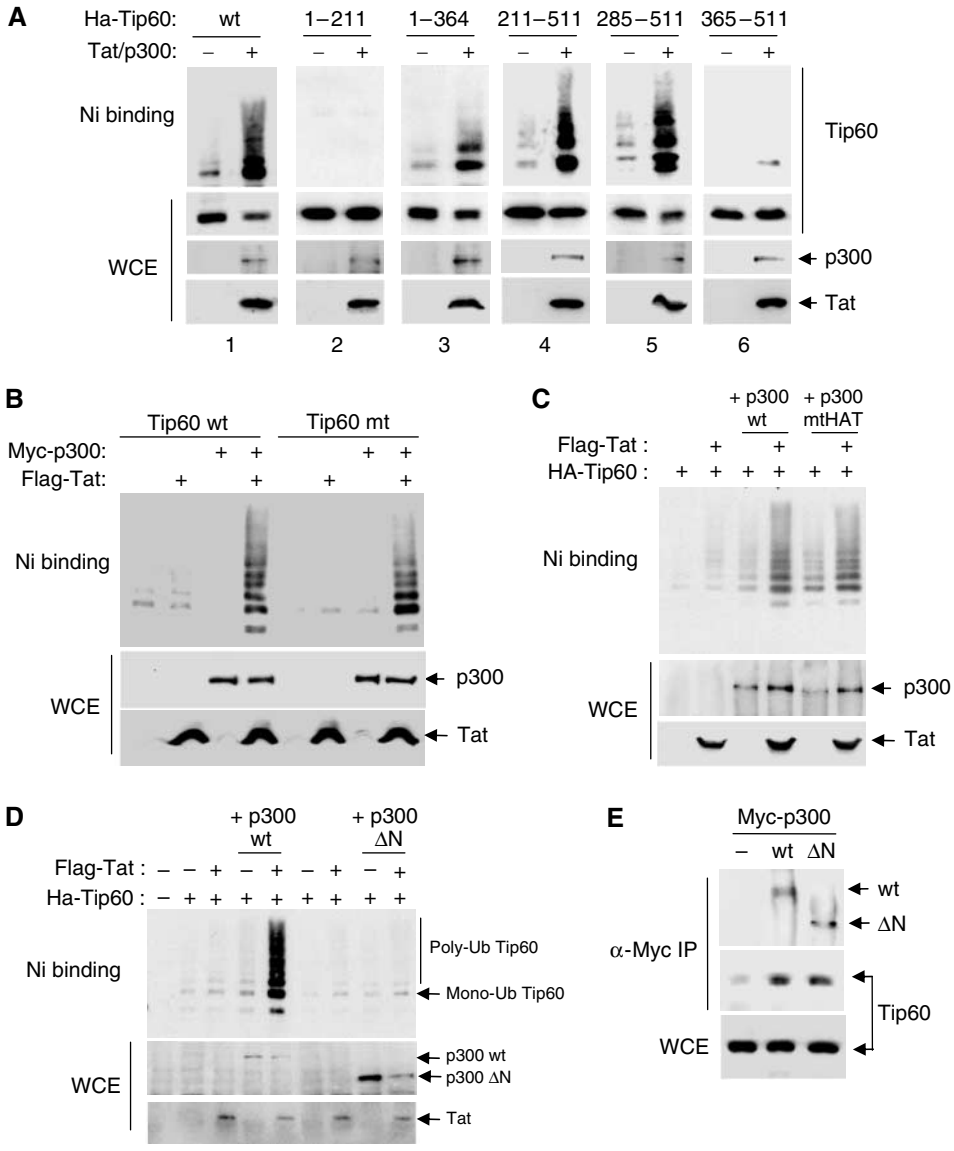

Figure 5 The stimulation of Tat/p300-mediated ubiquitination of Tip60 is acetylation independent. (**A**) The Tat/p300-dependent ubiquitination of wild-type Tip60 or the indicated mutants was monitored by nickel capture as described in Figure 1B. (**B**) The ubiquitination of the wild-type Tip60 (wt) or the K268/282R mutant (Tip60 mt) was monitored as above in the presence of the indicated proteins (+). (**C**) Tip60 ubiquitination was monitored in the presence of the indicated proteins. p300 mtHAT is the full-length protein containing an inactive HAT domain. (**D**) Tip60 ubiquitination was monitored as described, in the presence of the indicated proteins. p300 ΔN lacks the N-terminal 870 amino acids. (**E**) Ha-Tip60 and the indicated mutants of p300 (Myc-tagged) were coexpressed in Cos cells. After the immunoprecipitation of p300 proteins, the presence of associated Tip60 was assessed using an anti-Ha antibody. WCE shows the amount of Tip60 expressed in the cells.

priate siRNA, and cells were treated with different doses of Act D. Figure 6B shows that, as expected, the downregulation of Tip60, even partial, in H1299 cells was sufficient to induce a significant resistance to Act D-induced apoptosis.

These results therefore revealed a direct involvement of Tip60 in the apoptotic cell response to Act D treatment, and further showed that this particular set of Tip60-associated functions is annihilated in Tat-expressing cells.

To complete these investigations, we studied Tip60 expression and the apoptotic cell response to an Act D treatment in Jurkat cells infected with HIV-1. The use of an anti-Tip60 antibody showed a clear Tip60 downregulation in infected cells compared to noninfected control cells (Figure 7A). In order to evaluate the cell apoptotic response to an Act D treatment, we monitored the cleavage of poly-ADP-ribose polymerase (PARP) in control and infected cells treated with increasing amounts of Act D. Indeed, upon activation of cell apoptosis, PARP is known to be cleaved to generate at least two proteolytic fragments of 89 and 24 kDa (Soldani and Scovassi, 2002). Figure 7B shows a less efficient cleavage of PARP in the infected cells than in the noninfected control cells in response to Act D treatment, clearly demonstrating that HIV-1 infection confers to cells a significant resistance to genotoxic treatments.

Based on these results, we propose that the Tip60 neutralization (inactivation and downregulation) by Tat is a key event involved in the capacity of the viral protein to protect cells against DNA damage-induced apoptosis.

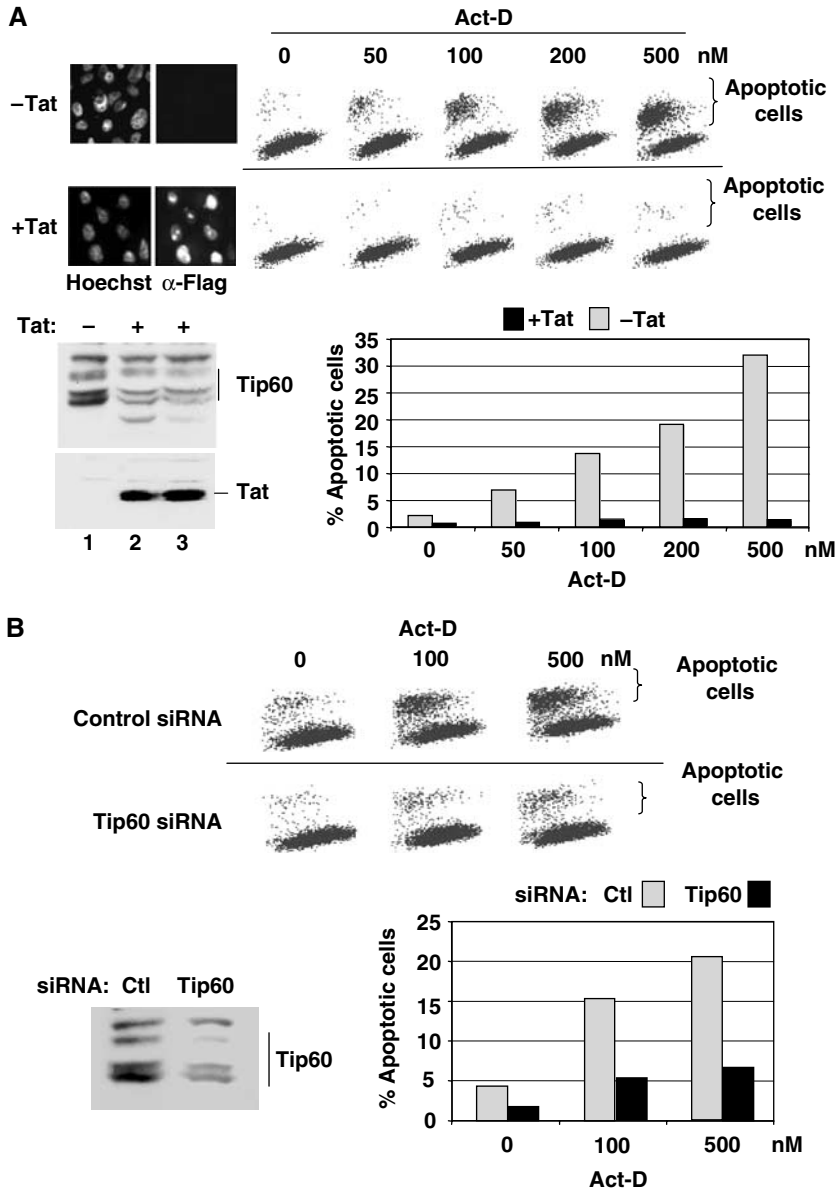

Figure 6 Targeting of Tip60 by Tat impairs apoptotic cell response to Act D treatment. (**A**) Nuclear extracts from control or two different Tat-expressing H1299 cell lines were analysed by anti-Tip60 and anti-Flag, detecting Tat (lower panels). The upper panel shows that Tat-expressing cells homogenously express the viral protein. Control or Tat-expressing H1299 cells were treated with the indicated concentrations of Act D for 16 h and the apoptotic cells visualized by immunofluorescent detection of the active caspase 3 and FACS analysis (right panel). The cell population containing active caspase 3 is indicated (apoptotic cells). The histogram indicates the % of cells containing active caspase 3 with respect to the total number of analysed cells (lower panel). (**B**) H1299 cells transfected with siRNA specifically downregulating Tip60 or control siRNA were treated with Act D, and apoptotic cells visualized as above (right panel). The effect of an siRNA treatment on Tip60 expression is shown in the left panel.

Discussion

The HIV-1 transactivator Tat (Jeang *et al*, 1999) has recently been shown to be ubiquitinated by Mdm2 (Bres *et al*, 2003). However, this ubiquitination did not lead to its degradation by the proteasome, but rather enhanced its transcriptional activity (Bres *et al*, 2003) probably through a facilitated recruitment of P-TEFb and a subsequent increase in RNA pol II processivity (Kurosu and Peterlin, 2004). Here we show that Tat, besides being ubiquitinated itself, is also able to use the cellular protein ubiquitination machinery to induce poly-ubiquitination of a specific cellular factor, Tip60. The analysis of the underlying mechanism has revealed new aspects of the cellular Tip60 regulation and highlighted the existence of additional similarities between the regulatory mechanisms controlling Tip60 and p53 concentrations.

A recent report has shown that p300/CBP possesses the ability to induce p53 polyubiquitination and is thereby involved in its downregulation (Grossman *et al*, 2003). This particular activity of p300/CBP is independent of its HAT activity and is contained within its N-terminal region. Here, we show that the same HAT-independent activity of these proteins is involved in the polyubiquitination of Tip60. Moreover, like p53, Tip60 was found to interact with p300/CBP, which could also acetylate the protein on two specific lysines. However, at this point, the regulation of Tip60 and

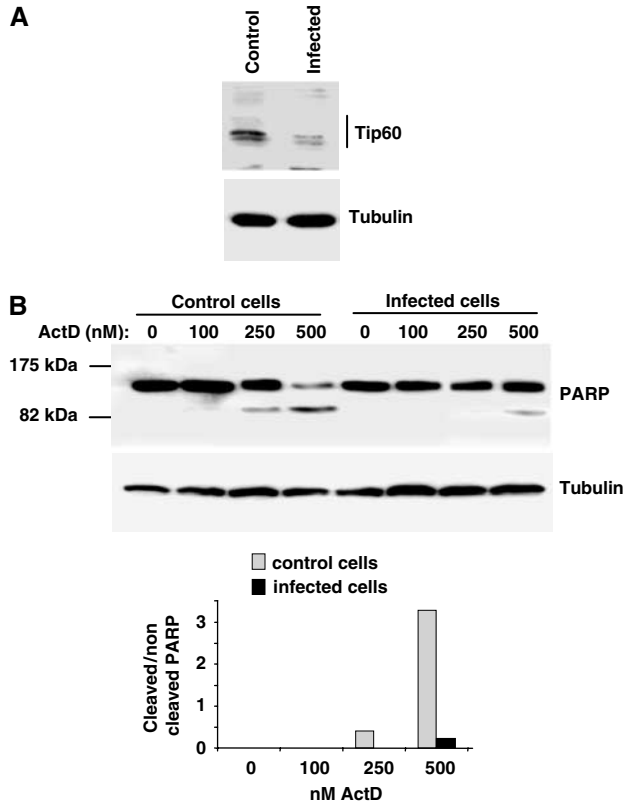

Figure 7 Tip60 downregulation and increased resistance to genotoxic stresses in HIV-1-infected Jurkat cells. (**A**) Jurkat cells were infected with HIV-1 and, 12 days after infection, extracts were prepared from infected and control (noninfected) cells. They were then probed with the anti-Tip60 and anti-tubulin antibodies. (**B**) Infected and noninfected control Jurkat cells were treated with the indicated amounts of Act D and the cleavage of PARP was monitored by Western blot (upper panel). Tubulin was revealed on the same blot as a control. The cleavage of PARP was quantified after the densitometric measurement of Western signals. The histogram shows signal value ratios of the 89 kDa PARP cleavage product to that of the noncleaved protein.

p53 diverges, since, in contrast to p53 (Ito *et al*, 2002; Li *et al*, 2002), our data show that Tip60 acetylation is not involved in controlling its ubiquitination, and a role for Tip60 acetylation, shown here to occur on its putative zinc-finger, was not found. Considering the available data on other acetylated nuclear factors, it is possible that Tip60 acetylation regulates the formation of Tip60-containing complexes (Doyon and Cote, 2004). Indeed, this particular domain of the protein is interacting with at least one partner, HDAC7 (Xiao *et al*, 2003).

The exact mechanism through which p300 and Mdm2 cooperate to stimulate Tip60 ubiquitination and the way Tat uses them remains to be established. p300 may have a genuine E4-type ubiquitin-ligase enzymatic activity, or it could alternatively serve as a nonenzymatic scaffold-type E4, interacting with both Tip60 and MDM2, similarly to YY1 in securing the p53/MDM2 interaction as reported by Shi and co-workers (Sui *et al*, 2004). Tat, through its capacity to interact with different components of this complex (Tip60, p300 and Mdm2), may act as an additional stability factor and further enhance enzyme–substrate interaction.

The recent discovery of the involvement of Tip60 in DNA repair and apoptosis in response to DNA damage (Ikura *et al*, 2000; Kusch *et al*, 2004) implies that by targeting Tip60, HIV-1 Tat could specifically alter the cell response to a genotoxic treatment. Here, we show that, in Tat-expressing or HIV-1-infected cells, the presence of DNA damage does not lead to caspase activation, indicating that Tat disrupts the link between DNA damage and cell apoptosis. We had previously found that Tat efficiently inhibits the HAT activity of Tip60 (Creaven *et al*, 1999). According to data published by Kusch *et al* (2004), this sole function of Tat could be sufficient to disturb the cell response to DNA damage, since the acetylation of the *Drosophila* γH2AX homolog by dTip60 was found to be necessary for its removal and replacement. The importance of Tip60 HAT activity is also strongly supported by data published by Ikura *et al* (2000) showing that the overexpression of a catalytically dead Tip60 mutant impairs apoptotic cell response to DNA damage.

Here, we show that, besides Tip60 inactivation, Tat also uses a unique cellular protein ubiquitination machinery to partially deplete the cells of Tip60. Although it is not clear why Tat uses two different mechanisms to neutralize Tip60 (inhibiting its catalytic activity as well as inducing its degradation), these data strongly suggest that Tip60 is a highly undesirable cellular protein for HIV-1. Since the amount of Tat could be very low in a cell, one possible explanation for the double effect of Tat on Tip60 could be that the induced Tat-dependent degradation of Tip60 would first lower the concentration of Tip60 to amounts that could then be catalytically inactivated by Tat.

The apoptosis of HIV-infected and bystander cells is thought to be the major cause of T-cell loss (Selliah and Finkel, 2001; Bell and Dockrell, 2003). However, emerging data suggest that HIV also developed mechanisms blocking and delaying the programmed cell death, increasing cell survival as well as activating cell proliferation (Fackler and Baur, 2002; Choi and Smithgall, 2004) with a particularly important role for Tat (Clark *et al*, 2000; Corallini *et al*, 2002; Deregibus *et al*, 2002; Chauhan *et al*, 2003; Bergonzini *et al*, 2004; Chipitsyna *et al*, 2004). In fact, it may be beneficial for HIV to inhibit apoptosis until high levels of progeny viruses are produced (Selliah and Finkel, 2001). Accordingly, it has been reported that, while Tat induces apoptosis in uninfected T cells in peripheral blood, it decreases apoptosis in HIV-1-infected T cells (McCloskey *et al*, 1997; Lum and Badley, 2003). In fact, the loss of the G_1/S checkpoint may provide a selective advantage for HIV by allowing virus transcription and virion production, which require T-cell cycling (Clark *et al*, 2000). Tat appears to play an important role in this particular process by inactivating the p53 pathway. HIV-1-infected cells have indeed lost the $p21^{cip1}$ gene expression, and this is accompanied by a loss of the G_1/S checkpoint (Clark *et al*, 2000). Moreover, p53 was shown to inhibit HIV-1 LTR transcription, and Tat could repress p53 gene expression (Duan *et al*, 1994; Li *et al*, 1995; Clark *et al*, 2000).

Our data show new aspects of the mechanisms used by Tat to inactivate the apoptotic cell response to DNA damage. Tip60 appears as an important cofactor for the p53-dependent p21 gene expression (Berns *et al*, 2004; Legube *et al*, 2004). Here, we show that Tat is capable of giving cells a remarkable resistance to genotoxic treatments. A role for Tat in increasing cell survival has already been reported (Corallini *et al*, 2002; Deregibus *et al*, 2002; Chauhan *et al*, 2003; Bergonzini

et al, 2004; Chipitsyna *et al*, 2004). Studies have shown that Tat-expressing PC12 cells efficiently resist gamma irradiation and cisplatin treatment (Chipitsyna *et al*, 2004; Choi and Smithgall, 2004) and that Tat promotes their serum-independent growth, colony formation in soft agar and accelerates tumour growth in nude mice (Bergonzini *et al*, 2004).

The protective and antiapoptotic effect of Tat becomes particularly important in the Kaposi's sarcoma (KS) development. Indeed, several studies have reported a primary role for Tat in the induction and development of KS in patients affected by AIDS. A dysregulation of cellular proliferation and a resistance to apoptosis are critical events favouring the growth and diffusion of KS, and Tat was found to be the survival factor for KS and endothelial cells (Cantaluppi *et al*, 2001). Our data strongly suggest therefore that the targeting of Tip60 by Tat is a critical event in giving Tat its survival-inducing role.

The data reported here also highlight a new property of Tat as a factor capable of interfering with cellular signalling by protein ubiquitination. The situation appears quite similar to the way Tat uses protein acetylation signalling to the benefit of the virus. By interacting with cellular HATs, Tat takes over the cellular acetylation signalling to optimize its transcriptional activity and to control acetylation-dependent cellular functions (Caron *et al*, 2003; Ott *et al*, 2004). Similarly, by interacting with Mdm2, Tat becomes ubiquitinated and moreover, as shown here, uses the Mdm2 system to ubiquitinate at least one cellular factor with the help of p300/CBP.

These findings therefore not only have shed new light on the functional relationship between critical cellular HATs, Tip60 and p300/CBP, but also revealed that HIV-1 has the potential of taking over an important cellular signalling system governed by protein ubiquitination with probably many important implications in controlling various ubiquitination-dependent cellular functions.

Materials and methods

Plasmids

pcDNA Ha-tagged Tip60 and deletion mutants have been described previously (Legube *et al*, 2002; Lemercier *et al*, 2003). Other mutants were constructed by PCR and inserted into pcDNA3-Ha. The point mutations were generated by PCR and controlled by DNA sequencing.

pSGHis-ubiquitin, pflag-Tat101 and pCMVNeoBam-Mdm2 were kind gifts from Dr D Bohman, Dr M Benkirane and Dr B Vogelstein, respectively. pSG-Myc p300 wt and mutants, kindly provided by Dr HG Stunnenberg, are described by Mitsiou and Stunnenberg (2003). pGal4-CBP wt and mutants were kind gifts from Dr T Kouzarides and are described by Martinez-Balbas *et al* (1998).

Cell culture, transfection, infection and treatments

Cos7, Jurkat and H1299 cells were cultured in Dulbecco's modified Eagle's medium (Cos7) and RPMI1640 medium (H1299 and Jurkat) supplemented with antibiotics and 10% fetal calf serum (FCS).

H1299 cells stably expressing Flag-Tat were established after transfection with pcDNA-Flag-Tat or empty vector and selection of cells in G418-containing medium. The pool of G418-resistant cells was then used to isolate individual Flag-Tat-expressing clones.

A total of 10^6 Jurkat cells were infected for 4 h at 37°C with 40 ng/ml of a virus suspension (strain X4: PNL4.3, kindly provided by Dr Yea Lih Lin and Dr Corbeau). The cells were pelleted by centrifugation, washed three times in 50 ml PBS and seeded in 40 ml RPMI (Invitrogen) complemented by 10% FCS (Cambrex). Every 3–4 days, the cells were seeded at 0.25×10^6 cells/ml. Cell infection was controlled by estimating the p24 production using a dedicated Elisa kit (Beckman Coulter) at days 0, 4, 7 and 12. At day 12, 10^7 cells were treated with Act D for 16 h and lysed in 100 µl of a lysis buffer (50 mM Tris pH 8, 120 mM NaCl, 5 mM EDTA and 0.5% NP-40). The concentration of soluble proteins was measured, and the extracts were used for Western blot analysis.

Plasmid transfections were performed with Fugene 6 reagent (Roche). The amounts of expression vectors were normalized with the corresponding empty vectors.

For siRNA treatment, cells were plated in six-well plates (10^5 cells/well), cultured for 24 h at 37°C and transfected twice (with a gap of 24 h) with 10 µl of 0.02 mM siRNA using oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. hp300 siRNA (CAGAGCAGUCCUGGAUUAG-dTdT, purchased from Eurogentec) and Tip60 siRNA (Tip1: ACGGAAGGUGGAGGUG GUU-dTdT, purchased from Dharmacon) were previously shown to decrease efficiently the level of corresponding mRNA or endogenous proteins (Bres *et al*, 2003; Legube *et al*, 2004). The control siRNA (GAAGUGUGCUGUACCCCAC-dTdT; Eurogentec) was directed against mouse HDAC6, and inefficient against HDAC6 in human cell lines used here (not shown).

Tip60 acetylation was enhanced by a 6 h treatment of cells with 50 ng/ml of TSA before their lysis.

Protein translation inhibition was achieved by treating the cells, 24 h post-transfection, with 30 µg/ml of CX (Sigma).

Apoptosis was induced by treating control or Tat-expressing H1299 cells with various concentrations of Act D for 16 h. Apoptotic cells were then visualized by FACS after the detection of active caspase 3 using a BD Pharmingen kit, according to the vendor's instructions.

Nickel capture

Cos cells were plated in 10 cm dishes and transfected with 4 µg of pSGHis-ubiquitin, 2 µg of pflag-Tat101 and/or 6 µg of pMyc-p300 wt or mutants (or pHa-CBP, or pCMVNeo/Bam Mdm2). Cells were lysed 24 h after transfection in 1 ml of buffer A (6 M guanidium-HCl, 100 mM Na_2HPO_4/NaH_2PO_4 pH 8.0 and 10 mM imidazole). Lysates were sonicated (100 J) to reduce the viscosity. After centrifugation, extracts were incubated with 50 µl of nickel-NTA-agarose beads (Qiagen) for 3 h at room temperature. Beads were then washed twice in buffer A, twice in the same buffer diluted five times in 50 mM Tris pH 6.8 and 20 mM imidazole and twice in 50 mM Tris pH 6.8 and 20 mM imidazole. Beads were then eluted with 30 µl of 2 × Laemmli sample buffer supplemented with 20 mM imidazole, and supernatants were subjected to SDS–PAGE and Western blotting.

Immunoprecipitation, cell fractionation and antibodies

Cos cells were transfected with 3 µg of Tip60 wt or mutants and 8 µg of pGal4-CBP and lysed 24 h after transfection by incubation for 1 h on ice in LSDB500 (500 mM KCl, 20% glycerol, 3 mM $MgCl_2$ and 50 mM Hepes pH 7.9) containing 0.2% NP-40, 1 mM DTT, 200 µg/ml DNaseI, 100 µg/ml RNaseA, 50 ng/ml TSA and protease inhibitors cocktail (complete mini EDTA-free; Roche). After centrifugation, the lysate was incubated with 1 µg of anti-Ha antibody (high-affinity 3F10; Roche) for 1 h on ice. Protein G-Sepharose beads (Amersham) were then added and incubated at 4°C on a rotoshake for 1 h. After three washes with LSDB250 (same as the lysis buffer with 250 mM KCl), complexes were recovered by adding Laemmli sample buffer and analysed by Western blots.

H1299 cells (control, Tat-expressing or treated with siRNAs) were lysed in buffer D (15 mM NaCl, 60 mM KCl, 12% sucrose, 2 mM EDTA, 0.5 mM EGTA, 0.65 mM spermidine, 1 mM DTT, 0.5 mM PMSF and 0.1% Triton X-100). Nuclei were pelleted by centrifugation and the supernatant (cytoplasmic extracts) was kept. Nuclei were resuspended in a small volume of buffer D and layered over 11 ml of the same buffer and centrifuged at 1000 *g* for 5 min. The pellet was lysed directly in protein loading buffer and homogenized by sonication (nuclear extracts).

Antibodies used are as follows: anti-Ha (Y-11; Santa-Cruz), anti-Gal4 (RK5C1; Santa Cruz), anti-acetylated lysine (Komatsu *et al*, 2003), anti-FlagM2 and anti-Myc (9E10; Sigma), anti-lamin (Santa-Cruz), anti-Mdm2 (SPM14; Santa-Cruz), anti-p300 (SantaCruz), goat anti-Tip60 (SantaCruz), rabbit anti-Tip60 (kindly provided by B Amati) and anti-PARP (PharMingen).

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